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# Differential effects of mineralocorticoid and angiotensin II on incentive and mesolimbic activity

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1  
2 **DIFFERENTIAL EFFECTS OF MINERALOCORTICOID**  
3 **AND ANGIOTENSIN II ON INCENTIVE AND MESOLIMBIC ACTIVITY**

4  
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10  
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19 Laura A. Grafe co-designed the research, executed the experiments, analyzed the data, and co-  
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21 wrote the paper.

22

23 ABSTRACT

24           The controls of thirst and sodium appetite are mediated in part by the hormones  
25 aldosterone and angiotensin II (AngII). The present study examined the behavioral and  
26 neural mechanisms of altered effort-value in animals treated with systemic  
27 mineralocorticoids, intracerebroventricular AngII, or both. First, rats treated with  
28 mineralocorticoid and AngII were tested in the progressive ratio operant task. The  
29 willingness to work for sodium versus water depended on hormonal treatment. In  
30 particular, rats treated with both mineralocorticoid and AngII preferentially worked for  
31 access to sodium versus water compared with rats given only one of these hormones.  
32 Second, components of the mesolimbic dopamine pathway were examined for  
33 modulation by mineralocorticoids and AngII. Based on cFos immunohistochemistry,  
34 AngII treatment activated neurons in the ventral tegmental area and nucleus accumbens,  
35 with no enhancement by mineralocorticoid pretreatment. In contrast, western blot  
36 analysis revealed that combined hormone treatment increased levels of phospho-tyrosine  
37 hydroxylase in the ventral tegmental area. Thus, mineralocorticoid and AngII treatments  
38 differentially engaged the mesolimbic pathway based on tyrosine hydroxylase levels  
39 versus cFos activation.

40

41 Key words: Aldosterone; Angiotensin; Dopamine; Motivation; Nucleus Accumbens; Sodium  
42 Appetite; Thirst; Ventral Tegmental Area

43

44

45 **Introduction**

46

47 Fluid depletion can be life threatening, and animals must carefully titrate their  
48 intake of water and sodium to restore and maintain osmotic and volemic balance.  
49 Sodium replacement requires the goal-directed behavior known as sodium appetite  
50 (Andersson, 1977), a behavior that can be prompted by mineralocorticoids, such as  
51 aldosterone, and angiotensin II (AngII) (Johnson and Thunhorst, 1997). AngII acts in the  
52 brain to elicit water intake and sodium intake (Epstein et al., 1969). During fluid depletion,  
53 suppression of either central AngII or aldosterone action does not eliminate sodium  
54 appetite, but blocking the central actions of both hormones abolishes the behavior (Buggy  
55 and Jonklaas, 1984; Sakai et al., 1986). Conversely, when both aldosterone and AngII  
56 are given exogenously, sodium appetite is potentiated (Fluharty and Epstein, 1983). The  
57 behavioral and neural basis for the combined effect of aldosterone and AngII on sodium  
58 appetite remains undefined.

59 The behavioral effects aldosterone and AngII to promote sodium ingestion may  
60 involve parallel behavioral mechanisms. For example, sodium ingestion could be  
61 enhanced by a change in the hedonic strength of the sodium tastant. Indeed, in rats  
62 placed on a sodium deficient diet, which increases aldosterone and AngII levels, sodium  
63 ingestion is preferred to moderately reinforcing brain stimulation, which suggests sodium  
64 appetite involves the modulation of the pleasurable properties of sodium intake (Conover  
65 et al., 1994). In parallel to altered taste value, sodium appetite may involve a recalibration  
66 of incentive-based effort. In this regard, rats treated with both aldosterone and AngII run  
67 faster on a runway to gain access to sodium, compared with rats treated with either

68 hormone alone (Zhang et al., 1984), which suggests that the combined hormone  
69 treatment increases the incentive value of sodium. The progressive ratio task is a  
70 quantitative assay for incentive-based effort, but the effects of combined  
71 mineralocorticoids and AngII on this behavioral test have not been reported.

72 The mesolimbic dopamine system has been widely implicated in effort-related  
73 behaviors (Barbano and Cador, 2006; Floresco et al., 2008; Kelley et al., 2005; Phillips  
74 et al., 2007; Salamone et al., 2009). Dopamine neurons in the ventral tegmental area  
75 (VTA) project to the accumbens, which in turn projects to brain regions such as the ventral  
76 pallidum to generate goal directed movement (Carelli, 2002). Previous work has  
77 implicated this brain system in sodium appetite. For example, the accumbens receives  
78 multisynaptic input from aldosterone-sensing and sodium-sensing neurons in the  
79 hindbrain (Miller and Loewy, 2014; Shekhtman et al., 2007). Sodium depletion alters the  
80 level of dopamine transporters and opioid peptides in the accumbens (Grondin et al.,  
81 2011; Lucas et al., 2003; Roitman et al., 1999). In addition, sodium depletion modifies  
82 the dendritic arbor of ventral striatum neurons (Roitman et al., 2002). Although these  
83 studies have suggested a role for mesolimbic activity in sodium appetite, the separate  
84 effects of aldosterone and AngII on mesolimbic activation have not been studied.

85 The present studies tested the overall hypothesis that mineralocorticoids and AngII  
86 recalibrate the willingness to work for sodium versus water. In particular, the willingness  
87 to work was measured with the progressive ratio task. In addition, the activity of the  
88 mesolimbic dopamine pathway was assessed with immunohistochemistry for cFos and  
89 western blot analysis for tyrosine hydroxylase. Although it is recognized that sodium  
90 depletion is a complex physiological state that is imperfectly mimicked with

91 mineralocorticoid and central AngII treatments, this preparation has yielded useful  
92 insights into the neuroendocrine actions that influence motivated behavior.

93

## 94 **Materials and Methods**

### 95 *Animals*

96         Adult male Sprague-Dawley rats (weight between 225 and 250 g) were obtained  
97 from Charles River Laboratories (Wilmington, MA, USA). Rats were pair-housed in plastic  
98 tubs with standard bedding and with food and water available *ad libitum*, except during  
99 experimental procedures. The temperature in the colony was maintained at 22°C with a  
100 12:12 h reversed light/dark cycle. Behavioral testing, described below, was conducted  
101 during the lights-out phase. Animals were allowed at least one week to acclimate to the  
102 colony before any procedures were performed. The Institutional Animal Care and Use  
103 Committee of the University of Pennsylvania approved all procedures with animals.

104

### 105 *Surgery*

106         Surgeries were performed in aseptic conditions. Animals were anesthetized with  
107 inducted and maintained with isoflurane anesthesia for stereotaxic surgery. A 26-gauge  
108 guide cannula (Plastics One, Roanoke, VA, USA) was aimed at the lateral ventricle using  
109 these coordinates: 0.48 mm caudal to bregma, 1.6 mm from mid-line and 4.2 mm ventral  
110 to dura mater. The cannulae were fixed in place with dental cement and bone screws.  
111 Upon completion of surgery procedures, animals were injected with yohimbine (0.11

112 mg/kg, ip, Ben Venue Laboratories Bedford, OH), and upon awakening animals were  
113 returned to the housing facility and singly housed. The animals were allowed at least five  
114 days to recover before verification procedures were performed.

115 Prior to undergoing experimental treatments, animals were tested for correct  
116 lateral ventricle cannula placement and patency. They were given an *icv* injection of 20.0  
117 ng of AngII diluted in artificial cerebrospinal fluid (aCSF) via a Hamilton syringe connected  
118 with PE-10 tubing to an injector that terminated 1 mm beyond the guide cannula. Animals  
119 were excluded from the experiment if they failed to demonstrate a drinking response in  
120 less than 30 seconds, consuming at least 3 ml of water, in two separate AngII challenges.  
121 Experiments began three days after these *icv* test injections.

122

### 123 *Experimental Design*

124 In all experiments, animals were assigned to one of four treatments in a 2 x 2  
125 design, with a crossover in behavioral experiments. Animals were first pre-treated twice  
126 daily (10 hours apart) for three days with a subcutaneous injection of sesame oil or an  
127 aldosterone analog, deoxycorticosterone acetate (DOC; 0.25 mg/0.2 ml sesame oil;  
128 Sigma, St Louis, MO). DOC penetrates the blood brain barrier more easily than  
129 aldosterone due to its low capacity for hydrogen bond formation (Kraulis et al., 1975 ).  
130 Animals then were injected *icv* with either artificial cerebrospinal fluid (aCSF; R&D  
131 Systems, Minneapolis, MN) or 20.0 ng AngII in a volume of 2.0 ul (Bachem, King of  
132 Prussia, PA). The treatment groups will be referred to as follows: Veh/Veh, DOC/Veh,  
133 Veh/AngII, DOC/AngII. Using this experimental design and identical doses, the

134 DOC/AngII treatment has been shown to elicit a greater than additive effect on sodium  
135 intake, but not water intake (Grafe et al., 2014).

136

### 137 *Experiment 1. Progressive Ratio Task*

138 Rats were acclimated to wire mesh cages for one hour with two 25-ml bottles  
139 containing tap water and 3% saline, each marked with 0.2 ml graduations. These bottles  
140 were then removed, and rats were water restricted for 23 hours per day for the next six  
141 days. During these six days, rats underwent operant lever pressing training in  
142 conditioning boxes for 30 minutes per day (Med Associates; MDPC IV Software, St.  
143 Albans, Vermont). The conditioning boxes contained levers for both water (right lever)  
144 and 3% saline (left lever), both simultaneously present. A lever press lowered a syringe  
145 pump, which delivered a 0.1 ml drop of the appropriate liquid into a cup available to the rat. The  
146 saline and water each had their own syringe pump and their own cup. During the first two  
147 training days, to facilitate learning, an aliquot was dispensed every 300 sec that elapsed  
148 without bar pressing. In addition, animals could earn a 0.1 ml of water or saline for each  
149 bar press, depending on which of the two levers was pressed. During the subsequent  
150 two training days, the animals earned a 0.1 ml water or saline for each lever press,  
151 followed by two training days during which three lever presses were required for each  
152 aliquot of water or saline. Animals were considered to have learned the lever-fluid  
153 contingencies when they had made at least 10 lever presses for water during the 30-min  
154 session. Once this occurred, rats were given ad libitum access to water again. Rats were  
155 then assigned to treatment groups, as described above, and given no further operant



156 training while they received their three days of pretreatment injections (vehicle or DOC).  
157 After pretreatment was complete, 24 hours after the last DOC treatment, rats were  
158 administered their assigned *icv* injection (vehicle or 20 ng AngII), and immediately given  
159 a test with a progressive ratio (PR) schedule. Thus, animals were water replete at the  
160 beginning of the PR test. The response requirement of the PR schedule increased  
161 progressively for both saline and water, as previously described (Davis et al., 2011). The  
162 breakpoint for each animal was defined as the final reinforced bar pressing set that  
163 preceded a 10-minute period without earning a reinforcement, with a two-hour limit total.  
164 Food was not available during this task.

165

#### 166 *Experiment 2: Hormone-Induced cFos expression*

167 To observe brain activation after DOC and AngII treatments, rats were assigned  
168 to treatment groups, as explained above. Sixty minutes after the last *icv* injection, each  
169 rat was anesthetized with 50 mg/kg ketamine and 20 mg/kg xylazine, intraperitoneally.  
170 As discussed below, there were group differences in the effort for sodium versus water  
171 during the first few minutes of the progressive ratio task, making 60 minutes post-  
172 treatment a reasonable time to expect differences in cFos levels. Rats were perfused  
173 transcardially with 100 mL of heparinized saline followed by 200 ml 4% paraformaldehyde  
174 (Electron Microscopy Sciences, Fort Washington, PA). The brains were isolated, post-  
175 fixed in paraformaldehyde overnight at 4°C, and then submerged in 20% sucrose in 0.1  
176 M phosphate buffer for three days. Coronal sections were cut on a freezing microtome  
177 into three serial sets of 40-um-thick sections. These sections encompassed the VTA and

178 the shell and core of the accumbens. One set of sections from each animal underwent  
179 immunohistochemical staining and analysis; the other two sets of sections were  
180 preserved in cryoprotectant as reserve material.

181 Sections were washed in Tris-buffered saline (TBS; pH 7.4) and then incubated  
182 with a cFos antibody (1:500, sc-52, rabbit; Santa Cruz Biotechnology, Santa Cruz, CA) in  
183 TBS with 0.2% TritonX-100 and 3% normal donkey serum (Jackson ImmunoResearch;  
184 West Grove, PA) overnight at 4°C. After several washes, sections were incubated with a  
185 Biotin-SP-conjugated AffiniPure Donkey Anti-rabbit IgG (1:100, Jackson  
186 ImmunoResearch) in TBS with 0.2% TritonX-100 and 3% normal donkey serum for 2 hours  
187 at room temperature. After several washes, sections were incubated with the Vectastain  
188 ABC kit (Vector Laboratories, Burlingame, CA) for one hour. This was followed by another  
189 set of washes before staining with 3'3'-diaminobenzidine (Sigma-Aldrich) for 10 minutes.  
190 After a final set of washes, sections were mounted on slides, air-dried, and cover slipped  
191 with DPX mounting media (Electron Microscopy Sciences: Fort Washington, PA).

192 Photomicrographs were acquired with a digital camera (Diagnostic Instruments,  
193 Sterling Heights, MI, model RTKE), maintaining the same microscope and camera  
194 settings to ensure the same level of light and exposure for all images. Background was  
195 subtracted from images using Photoshop, and images were set to the same threshold  
196 level (200). Images were further analyzed in NIH Image J using standardized boxes for  
197 each brain region (based on Paxinos & Watson Rat Brain Atlas, shown in **Figure 1A**),  
198 using the analyze particles function, which counts objects within specific size and shape  
199 parameters. Pixel size minimum was 15 and circularity was set to 0.35.

200

201 *Experiment 3: Hormone Regulation of Tyrosine Hydroxylase*

202         Animals were assigned to four treatment groups, as described above. Five  
203 minutes after the last injections, animals were rapidly decapitated and brains were flash  
204 frozen in hexane over dry ice. The five-minute time point was selected based on its  
205 correlation with a significant increase in lever presses for sodium in the progressive ratio  
206 experiment. Using a cryostat, 1-mm punches of the brain regions of interest (VTA,  
207 accumbens shell and core, shown in **Figure 1B**) were collected from 300- $\mu$ m sections.  
208 Punches were immersed in lysis buffer containing 25 mM Tris-HCl (pH 8), protease  
209 inhibitors (pepstatin, leupeptin, aprotinin), and phosphatase inhibitors (sodium  
210 pyrophosphate, sodium fluoride, sodium molybdate, phenylarsin oxide, and sodium  
211 orthovanadate). The VTA and accumbens (shell and core) punches were immersed in  
212 50 and 100  $\mu$ L of lysis buffer, respectively. Brain punches were sonicated for three  
213 seconds followed by centrifugation at 14,000 rpm in 4°C for 15 minutes. Supernatant was  
214 collected and a Bicinchoninic acid (BCA) protein assay was performed on five microliters  
215 of each sample. Based on the protein levels detected by the BCA assay, appropriate  
216 amounts of sample and sample buffer were loaded into wells of a 10% sodium dodecyl  
217 sulfate polyacrylamide gel. Western blots for phospho- and total TH were performed  
218 using the LiCor Odyssey System. The following antibodies were used: monoclonal anti-  
219 tyrosine hydroxylase antibody T2928 at 1:8000 (Sigma Aldrich); tyrosine hydroxylase  
220 pS31 rabbit polyclonal antibody #36-9900 at 1:400 (Life Technologies, Carlsbad, CA);  
221 IRDye 800CW Goat anti-Mouse IgG (H + L) 926-32210 at 1:2000 (Li-Cor Biosciences,  
222 Lincoln, NE); IRDye 680LT Goat anti-Rabbit IgG (H + L) 926-68021 at 1:4000 (Li-Cor

223 Biosciences). Phosphorylation sites on tyrosine hydroxylase generally increase  
224 catecholamine production. Serine 31, the residue attended to in the present study, is  
225 phosphorylated by depolarization and extracellular receptor-activated kinases 1 and 2  
226 (Tekin et al., 2014).

227

## 228 *Statistical Analysis*

229 Data are presented as the mean  $\pm$  the standard deviation of the mean. For all  
230 experiments, comparisons were made between treatment variables with a two-way  
231 ANOVA. When warranted, Bonferonni-corrected t-tests were performed. Effect sizes  
232 were calculated using  $\eta^2$  and Cohen's d. All hypothesis tests used  $\alpha=0.05$  as the criterion  
233 level of significance. Statistical analyses were conducted using Prism 2.0 software (La  
234 Jolla, CA).

235

## 236 **Results**

237

238 The hormone regimen used in the present study was previously demonstrated to  
239 increase sodium ingestion in the Veh/AngII and DOC/AngII groups and to significantly  
240 water intake in the Veh/AngII condition (Grafe et al., 2014). Here, the progressive ratio  
241 operant task was used to compare the effort rats are willing to exert for sodium ingestion  
242 after these hormone treatments. Rats were pretreated with oil or DOC, followed by *icv*  
243 treatments of vehicle or AngII and allowed access to two levers: one for access to 3%  
244 saline and the other for access to water (0.1 ml per reinforcement). **Figure 2A** illustrates

245 the cumulative number of lever presses across the duration of testing for 3% saline and  
246 water. DOC/AngII treated rats bar-pressed for a longer duration for 3% saline compared  
247 with the other treatment groups.

248 We conducted separate two-way ANOVA tests to examine effects of these  
249 hormones on bar presses for sodium and water. The two-way ANOVA supported a main  
250 effect for AngII ( $F(1,29) = 13.9$ ,  $p = 0.0008$ ,  $\eta^2 = 0.31$ ) but not DOC ( $F(1,29) = 0.1$ ,  $p =$   
251  $0.752$ ,  $\eta^2 > 0.01$ ) on 3% saline lever presses. Bonferroni-corrected post-hoc t tests  
252 revealed that the DOC/AngII group pressed significantly more on the saline lever  
253 compared with the Veh/Veh and DOC/Veh groups (Veh/Veh: 10.9 presses (SD2.4),  
254 DOC/Veh: 6.4 presses (SD1.7), Veh/AngII: 19.1 presses (SD6.4), DOC/AngII: 26.0  
255 presses (SD2.8);  $p = 0.001$ ,  $p = 0.0001$ ;  $d = 1.50$ ). A two-way ANOVA established a main  
256 effect for AngII ( $F(1,29) = 5.7$ ,  $p = 0.023$ ,  $\eta^2 = 0.13$ ) but not DOC ( $F(1,29) = 1.7$ ,  $p = 0.203$ ,  
257  $\eta^2 = 0.04$ ) on water lever presses, and a significant interaction between the two hormones  
258 ( $F(1,29) = 8.4$ ,  $p = 0.007$ ,  $\eta^2 = 0.19$ ). Post-hoc analysis revealed that rats treated with  
259 Veh/AngII pressed more for water than any other treatment group (Veh/Veh: 16.9 presses  
260 (SD5.2), DOC/Veh: 29.5 presses (SD4.9), Veh/AngII: 58.9 presses (SD10.7),  
261 DOC/AngII: 25.5 presses (SD8.9);  $p = 0.003$ ,  $p = 0.020$ ,  $p = 0.030$ ;  $d = 1.47$ ).

262 With a progressive ratio schedule, the response requirement to attain rewards  
263 increases according to the same rule throughout the test session until the rat stops  
264 responding (Sclafani and Ackroff, 2003). The highest set of bar presses completed is  
265 referred to as the break point and provides a measure of incentive. In the present  
266 experiment, rats had simultaneous access to work for sodium and water. Treatments for  
267 Veh/Veh, DOC/Veh and Veh/AngII elicited more work for water compared with sodium,

268 whereas the DOC/AngII-treated animals exhibited the opposite pattern. A two-way  
269 ANOVA established an interaction between AngII and DOC ( $F(1,29) = 4.6, p = 0.046$ ;  
270  $F(1,29) = 8.6, p = 0.006; \eta^2 = 0.18$ ). DOC/AngII treated rats had significantly higher ratio  
271 of the breakpoint for sodium versus breakpoint for water than all other treatment groups.

272

### 273 *Experiment 2: Hormone-Induced cFos expression*

274 The observed unique effect of DOC/AngII treatment on willingness to work for  
275 sodium versus water suggested an underlying difference in mesolimbic activity. As  
276 shown in **Figure 3**, the number of cFos labeled cells was quantified in the VTA and the  
277 accumbens after Veh/Veh, DOC/Veh, Veh/AngII, and DOC/AngII treatment. A two-way  
278 ANOVA supported a main effect for AngII ( $F(1,13) = 39.94, p < 0.0001, \eta^2 = 0.64$ ), but  
279 not for DOC or an interaction between the two hormones in the VTA. Post hoc tests  
280 indicated that Veh/AngII and DOC/AngII treatments increased cFos expression compared  
281 with Veh/Veh ( $p = 0.0001, p = 0.0003$ , respectively;  $d = 3.77$ ). In the nucleus accumbens  
282 core, a two-way ANOVA revealed a main effect for AngII ( $F(1,13) = 12.82, p = 0.0034, \eta^2$   
283  $= 0.44$ ), but no effect for DOC or their interaction on cFos expression. Post-hoc tests  
284 indicated that Veh/AngII and DOC/AngII treatments increased cFos levels compared to  
285 Veh/Veh ( $p = 0.0004, p = 0.0046$ , respectively;  $d = 2.12$ ). Lastly, a two-way ANOVA for  
286 the nucleus accumbens shell revealed a main effect for AngII ( $F(1,13) = 18.6, p = 0.0008,$   
287  $\eta^2 = 0.52$ ), but no effect for DOC or the two-hormone interaction on cFos expression.  
288 Post-hoc t tests indicated that each AngII treatment group induced significant cFos  
289 expression compared to the Veh/Veh treatment (Veh/AngII  $p = 0.0012$ ; DOC/AngII  $p =$

290 0.002;  $d = 2.48$ ). To summarize, AngII treatment activated cFos expression in the VTA  
291 and nucleus accumbens, but DOC pretreatment did not enhance this activation.

292

### 293 *Experiment 3: Hormone Regulation of Tyrosine Hydroxylase*

294 The AngII-induced expression of cFos of the VTA observed in Experiment 2  
295 suggested a possible increase in dopamine neurotransmission. The level of tyrosine  
296 hydroxylase, the rate-limiting enzyme for dopamine production, and its phosphorylation,  
297 were measured by western blot analysis. Rats were pretreated with vehicle or DOC  
298 followed by *icv* injections of vehicle or AngII, and tissue punches were collected five  
299 minutes after the last injection. In the VTA, where dopaminergic neurons reside, a two-  
300 way ANOVA supported a main effect for both DOC and AngII on tyrosine hydroxylase  
301 levels in the VTA ( $F(1,8) = 16.6$ ,  $p = 0.004$ ,  $\eta^2 = 0.51$ ;  $F(1,8) = 6.0$ ,  $p = 0.04$ ,  $\eta^2 = 0.19$ ),  
302 as illustrated in **Figure 4A**. Post hoc tests indicated each treatment group significantly  
303 enhanced tyrosine hydroxylase levels compared with vehicle ( $p = 0.003$ ,  $p = 0.04$ ,  $p =$   
304  $0.02$  for DOC/Veh, Veh/AngII, and DOC/AngII, respectively;  $d = 1.99$ ). However, DOC  
305 pretreatment did not further enhance the effects of AngII on tyrosine hydroxylase levels.  
306 For phospho-tyrosine hydroxylase, the value for each animal, as determined by western  
307 blot analysis, was divided by the value for total tyrosine hydroxylase (p-TH/TH). In the  
308 VTA, a two-way ANOVA supported a main effect for DOC ( $F(1,8) = 10.37$ ,  $p = 0.012$ ,  $\eta^2$   
309  $= 0.52$ ), but not AngII treatment, as shown in **Figure 4B**. Post-hoc analysis indicated that  
310 in the VTA DOC/AngII groups displayed increased p-TH/TH levels compared with  
311 Veh/Veh ( $p = 0.02$ ;  $d = 0.78$ ).

312 In the nucleus accumbens core, neither DOC nor AngII had a significant main  
313 effect on tyrosine hydroxylase levels. In the shell of the nucleus accumbens, DOC, but  
314 not AngII, had a main effect on levels of tyrosine hydroxylase ( $F(1,8) = 9.3$ ,  $p = 0.02$ ,  $\eta^2$   
315  $= 0.40$ ). Post-hoc analysis revealed DOC/Veh increased tyrosine hydroxylase levels  
316 compared with Veh/Veh ( $p = 0.04$ ;  $d = 2.17$ ). In both the core and shell DOC and AngII  
317 treatments significantly increased p-TH/TH compared with vehicle (Core: Veh/Veh: 0.02  
318 (SD0.2), DOC/Veh: 0.42 (SD0.2), Veh/AngII: 0.37 (SD0.14), DOC/AngII: 0.38 (SD0.01),  
319  $p = 0.011$ ,  $p = 0.020$ ,  $p = 0.018$ ;  $d = 0.72$ ); (Shell: Veh/Veh: 0.07 (SD0.05), DOC/Veh:  
320 0.46 (SD0.02), Veh/AngII: 0.38 (SD0.06), DOC/AngII: 0.39 (SD0.04);  $p = 0.0008$ ,  $p =$   
321  $0.0028$ ,  $p = 0.0022$ , respectively;  $d = 0.51$ ). In summary, in both regions of the nucleus  
322 accumbens, DOC and AngII pretreatments increased phosphorylation levels of tyrosine  
323 hydroxylase, usually without detectably increasing total tyrosine hydroxylase levels.

324

## 325 **Discussion**

326

327 During sodium deficiency, aldosterone and AngII act in concert to produce an avid  
328 consumption of sodium (Fluharty and Sakai, 1995), and combined treatment with these  
329 hormones is a useful experimental model for investigating the biological basis of this  
330 striking example of motivated behavior. The current experiments tested the overall  
331 hypothesis that DOC and AngII treatments uniquely affect the willingness to work for  
332 access to sodium, potentially by modulating neural activation and neurochemistry in the  
333 mesolimbic dopamine pathway. DOC/AngII-treated rats steadily exerted effort for access  
334 to 3% sodium solution while decreasing their effort for access to water compared with the



335 Veh/AngII-treated rats. DOC and AngII had differential effects on cFos activation and  
336 phospho-tyrosine hydroxylase levels in the VTA. Additive effects of DOC and AngII were  
337 not observed when these treatments were combined, although it is possible that cFos  
338 immunohistochemistry and tyrosine hydroxylase immunoblots were limited by a ceiling  
339 effect. Instead, the co-treatment of DOC and AngII was associated with a unique  
340 combination of enhanced dopamine synthetic capacity and neural activation. **Figure 5**  
341 summarizes these results according to hormone treatment, brain region and behavior.

342 Several caveats are well known for the interpretation of cFos expression. First,  
343 neuronal activation may occur without cFos induction. Thus, the lack of cFos activation  
344 after DOC treatment may not reflect the recent activity of these neurons. In addition, the  
345 neural consequences of cFos activation are not fully understood, making cFos merely a  
346 marker of recent activity. Thirdly, without knowing the phenotype of the cFos labeled  
347 neurons in this study, the increased activity may occur in excitatory or inhibitory neurons,  
348 which would have quite different physiological consequences. With those points  
349 notwithstanding, cFos expression in the VTA and ventral striatum has been a valuable a  
350 proxy for measuring neuronal activation in many studies (Kovács, 2008).

351

### 352 *Behavioral mechanisms*

353 Healthy rats normally avoid concentrated sodium solutions (Berridge et al., 1984);  
354 however when levels of both mineralocorticoids and AngII are elevated, a robust sodium  
355 appetite emerges (Fluharty and Epstein, 1983; Shade et al., 2002). A component of  
356 sodium appetite is altered gustatory hedonic processing, and subsequent increased

357 pleasure, of passively received oral sodium. In the taste reactivity test, sodium appetite  
358 is associated with reduced gaping and increased pre-swallowing behaviors in response  
359 to orally presented sodium solutions (Berridge et al., 1984; Clark and Bernstein, 2006).  
360 Further evidence that sodium depletion increases the “liking” of concentrated sodium  
361 solutions is the depletion-specific response of neurons in the ventral pallidum, a brain  
362 region thought to encode pleasure, in response to sodium ingestion (Tindell et al., 2006).  
363 The ventral forebrain may contribute to the hormonal modulation of sodium as a tastant  
364 (Garcia et al., 2008; Geerling and Loewy, 2006; Lundy, 2008).

365         In addition to enhanced palatability, sodium appetite includes a willingness to work  
366 for sodium. For example, rats run faster along a runway to gain access to sodium after  
367 co-administration of aldosterone and AngII (Zhang et al., 1984). In the progressive ratio  
368 test, the highest set of bar presses completed, referred to as the break point, provides a  
369 quantification of goal value (Hodos, 1961). Progressive ratio schedules model foraging  
370 in a natural setting; as resources are consumed in a given area, more effort is required to  
371 obtain them. This method has been used to quantify the motivation for sodium after body  
372 fluid depletion (Clark and Bernstein, 2006; Starr and Rowland, 2006). The present  
373 experiment used a two-bottle progressive ratio task to document the effects of  
374 mineralocorticoids and AngII on the relative effort-value of 3% sodium solution versus  
375 water. This preparation revealed a shift in effort-value, with AngII alone biasing the  
376 animals to exert more effort for water than sodium, whereas DOC/AngII treatment drove  
377 them to work more for 3% saline than water. Unlike previous studies of sodium appetite  
378 that focused on the quantity of sodium ingested, the two-bottle progressive ratio task  
379 quantifies the competing drives for sodium and water without changing fluid balance.

380 Differences in effort-value led us to investigate effects of mineralocorticoids and AngII on  
381 mesolimbic regions, a brain system that regulates effort-related behaviors (Barbano and  
382 Cador, 2006; Floresco et al., 2008; Kelley et al., 2005; Phillips et al., 2007; Salamone et  
383 al., 2009).

384 In the present study, no simple correlation exists between the level of cFos  
385 activation in a specific brain region and behavior. Rather, similar cFos levels were  
386 observed in the VTA and nucleus accumbens when animals would have been working  
387 for water versus sodium. One interpretation of this finding is that cFos activation in the  
388 mesolimbic dopamine pathway represents a non-specific increase in willingness work.  
389 However, it remains uncertain whether the similar numbers of cFos labeled cells were of  
390 the same cell type. Furthermore, despite the similarities in cFos levels despite during  
391 these different motivated states, it should be noted that regulation of tyrosine hydroxylase  
392 varies, suggesting differential dopamine transmission. Thus, further studies are needed  
393 to understand the details of motivation-specific regulation of mesolimbic dopamine.

394

#### 395 *Hormone-sensing brain regions*

396 The initial brain targets of mineralocorticoids and AngII are known. Regarding  
397 mineralocorticoids, the nucleus of the solitary tract contains a unique population of  
398 neurons that co-express mineralocorticoid receptors and the enzyme necessary for  
399 mineralocorticoid-specific action, known as 11- $\beta$  hydroxysteroid dehydrogenase 2  
400 (Geerling et al., 2006; Geerling and Loewy, 2006). In contrast, humoral AngII acts initially  
401 on AngII type 1 (AT1) receptors in circumventricular organs, such as the subfornical organ

402 and the organ vasculosum of the lateral terminalis (Lind et al., 1985; McKinley et al.,  
403 1992b; Tanaka et al., 1986; Weiss and Hatton, 1990). Mineralocorticoids exert minor up-  
404 regulation of AT1 receptor levels and AT1 signaling (Grafe et al., 2014; Shelat et al.,  
405 1999a; Shelat et al., 1998; Shelat et al., 1999b). As a parallel mechanism,  
406 mineralocorticoids may exert a cooperative behavioral effect with AngII by acting  
407 elsewhere in a broader circuit. For example, mineralocorticoids appear to remove an  
408 inhibitory influence on sodium appetite, namely oxytocin activity in the PVN (Blackburn et  
409 al., 1992; Grafe et al., 2014; Roesch et al., 2001; Stricker and Verbalis, 1987).

410 A behaviorally relevant downstream connection of both mineralocorticoid- and  
411 AngII-sensing brain regions is the lateral hypothalamus (Camacho and Phillips, 1981),  
412 which is essential for the normal expression of sodium appetite (Dayawansa et al., 2011).  
413 The lateral hypothalamus, in turn, projects to the mesolimbic dopamine system via the  
414 medial forebrain bundle (Berk and Finkelstein, 1981; Saper et al., 1979). Relevant  
415 projections from the lateral hypothalamus to the VTA may include orexin neurons (Fadel  
416 and Deutch, 2002; Narita et al., 2006; Peyron et al., 1998), which promote incentive-  
417 based effort in the VTA (Borgland et al., 2009; Choi et al., 2010). Furthermore, stimulation  
418 of the lateral hypothalamus promotes sodium appetite (Liedtke et al., 2011). Likewise,  
419 AngII treatment enhances the release of striatal dopamine in freely moving rats within  
420 minutes, which then is associated with increased drinking behavior (Brown et al., 1996;  
421 Hoebel et al., 1994). Thus, a possible link between mineralocorticoid and AngII-sensing  
422 brain regions and midbrain dopamine neurons may be lateral hypothalamic orexin  
423 neurons. Future studies are needed to illuminate the details of this broader circuit.

424

425 *Sodium appetite and motivation systems*

426           The mesolimbic pathway is part of a motor loop that links the incentive properties  
427 of stimuli with the effort exerted to approach or avoid them (Berridge, 2007; Salamone et  
428 al., 2007). Neural adaptations in this brain region are associated with motivated  
429 behaviors (Gu et al., 2010). AngII-induced cFos expression in the mesolimbic area is  
430 likely the result of transynaptic activation because the VTA does not have AngII receptors  
431 (Song et al., 1992). Previous studies documented AngII-induced activation of the  
432 hypothalamus and taste nuclei (Han and Rowland, 1995; Houtp et al., 1998; McKinley et  
433 al., 1992a; Thunhorst et al., 1998; Vivas et al., 1995), but this is the first report of cFos  
434 activation in the VTA.

435           The VTA includes many dopaminergic neurons (Oades and Halliday, 1987). The  
436 AngII-induced increase in tyrosine hydroxylase levels supports the notion that dopamine  
437 neurons are activated by AngII treatment. A similarly rapid increase in tyrosine  
438 hydroxylase levels occurs during exposure to cocaine-paired contexts (Liang et al., 2012).  
439 AngII-induced cFos expression may contribute to the up-regulation of tyrosine  
440 hydroxylase (Gheea et al., 1998). DOC treatment markedly increased the  
441 phosphorylation of tyrosine hydroxylase, which augments catalytic activity of this rate-  
442 limiting enzyme (Dunkley et al., 2004). The combined actions of AngII and DOC on  
443 neuronal activation and tyrosine hydroxylase would be expected to produce a substantial  
444 increase in dopamine release at axonal targets. Future studies are needed to examine  
445 the consequences of these hormonal actions at downstream nodes in the motivation  
446 circuit.

447           Regarding the ventral striatum, neurons there exhibit neural plasticity during  
448 various motivational states, including depletion-induced sodium appetite (Roitman et al.,  
449 2002). As in the VTA, AngII treatment increased cFos levels without a detectable  
450 augmentation by DOC pretreatment. Unlike the VTA, all hormone treatments increased  
451 phosphorylation of tyrosine hydroxylase levels with detectable increases in total levels of  
452 this enzyme. It will be important for future studies to investigate the neurophysiological  
453 consequences and potential rewiring that may occur in the accumbens in response to  
454 AngII- versus DOC/AngII-induced changes in this dopamine pathway.

455

#### 456 *Conclusions*

457           In summary, the DOC/AngII-induced sodium appetite involves a shift in the effort  
458 value of sodium versus water consumption, noted in the break point for lever pressing.  
459 DOC and AngII affect the mesolimbic dopamine pathway differently, with AngII treatment  
460 increasing cFos activation in the VTA and nucleus accumbens, and DOC increasing  
461 phosphorylation of tyrosine hydroxylase. Thus, these two hormones may act in parallel  
462 to enhance mesolimbic dopamine transmission through complementary mechanisms.

463

464

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466

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696



697 **Figure Legends**

698

699 **Figure 1.** Drawings of rat brain coronal sections depicting the regions of interest for both  
700 cFos immunohistochemistry (**Panel A**) and tyrosine hydroxylase activation assays (**Panel**  
701 **B**). **Panel A.** Boxes surrounding each brain region represent the areas analyzed for  
702 cFos cell counts. **Panel B.** Circles within each brain region represent 0.75-mm  
703 micropunches collected for the tyrosine hydroxylase western blots. The brain regions  
704 examined include both the core and shell of the accumbens, as well as the VTA.  
705 Abbreviations: AcbC = Core of the accumbens, AcbS = Shell of the accumbens, CPu=  
706 caudate putamen, ml = medial lemniscus, LS = lateral septal nucleus, PAG =  
707 periaqueductal gray, VTA= ventral tegmental area.

708

709 **Figure 2.** DOC/AngII treatment increases relative motivation for sodium (n = 5-6/group).  
710 **Panel A.** Line graphs illustrating cumulative presses over time for 3% Saline and Water  
711 after Vehicle, DOC, AngII, or DOC plus AngII treatments. **Panel B.** Bar graphs illustrating  
712 total lever presses for 3% saline and water after Vehicle, DOC, AngII, or DOC plus AngII  
713 treatments. AngII-only treatment increased bar presses for water and sodium, whereas  
714 DOC plus AngII treatment mainly increased presses for sodium. **Panel C.** Bar graphs  
715 illustrating the breakpoint ratio of sodium to water after Vehicle, DOC, AngII, or DOC plus  
716 AngII. DOC plus AngII causes the highest sodium to water breakpoint ratio.  
717 Abbreviations: AngII= Angiotensin II, DOC = deoxycorticosterone acetate, Veh = vehicle.

718

719 **Figure 3.** DOC/AngII treatment increases cFos expression in the ventral tegmental area  
720 and the accumbens (n = 3-6 per group). Bar graphs illustrate the cFos cell counts in the  
721 ventral tegmental area and accumbens after either vehicle or DOC pretreatment followed  
722 by *icv* vehicle or AngII. Representative images of the VTA and accumbens (coronal  
723 plane, 10x) in each treatment condition are shown above the bar graphs. AngII and  
724 DOC/AngII treatment increased cFos expression in the VTA, with DOC/AngII inducing the  
725 most immunostaining. In the core and shell of the nucleus accumbens, each hormone  
726 treatment induced cFos immunostaining compared with vehicle; DOC/AngII induced the  
727 highest amount of cFos immunostaining. Asterisks indicate a significant increase  
728 compared with the Veh/Veh group. Abbreviations: AngII = Angiotensin II, DOC =  
729 deoxycorticosterone acetate, NuAcc= Accumbens, Veh = Vehicle, VTA= Ventral  
730 Tegmental Area

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732 **Figure 4.** DOC treatment increases tyrosine hydroxylase activation in the VTA and the  
733 accumbens (n = 3/group). **Panel A.** Bar graphs illustrate tyrosine hydroxylase levels in  
734 the VTA and the accumbens (core and shell) after oil versus DOC pretreatment and *icv*  
735 saline versus AngII. Representative western blot images of tyrosine hydroxylase are  
736 shown above each quantified bar. Each treatment increased tyrosine hydroxylase  
737 expression in the VTA. **Panel B.** Bar graphs illustrate phosphorylated tyrosine  
738 hydroxylase levels in the VTA and the accumbens (core and shell) after either oil or DOC  
739 pretreatment followed by *icv* treatments with AngII. Only DOC pretreatment significantly  
740 increased phosphorylated tyrosine hydroxylase expression compared to vehicle in the  
741 VTA and accumbens core. However, each treatment increased phosphorylated tyrosine

742 hydroxylase expression in the accumbens shell. Panel C. Representative western blot  
743 images labeled with corresponding treatment groups and brain regions assayed for  
744 tyrosine hydroxylase (left) and phosphorylated tyrosine hydroxylase (right).  
745 Abbreviations: AngII = Angiotensin II, Acc= Accumbens, DOC = deoxycorticosterone  
746 acetate phospho-TH = phosphorylated tyrosine hydroxylase, TH = tyrosine hydroxylase,  
747 Veh = Vehicle, VTA= ventral tegmental area. Asterisks indicate  $p < 0.05$  compared with  
748 the Veh/Veh group.

749

750 **Figure 5.** Diagram summarizing the effects of DOC and AngII on the mesolimbic system  
751 and behavior. Abbreviations: AngII = Angiotensin II, NuAcc= Nucleus Accumbens, DOC  
752 = deoxycorticosterone acetate, p-TH = phosphorylated tyrosine hydroxylase, TH =  
753 tyrosine hydroxylase, VTA= ventral tegmental area.

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